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14. ABSTRACT

Our main objective is to establish whether the growth factor called FGF that is found in the breast merits the development as a target in a new breast cancer therapeutic drug discovery program. We are investigating whether a "dysinhibition" model of breast cancer progression is correct by asking the question does changing FGF content in mammary cancer change cancer progression. If so, we would justify setting up drug screening initiative to discover agonists, if not antagonists would be the preferred target. To meet this objective we are exploiting a mouse model of spontaneous mammary tumor development and creating lines of mice that are cancer prone/resistant by engineering a lack one of the presumed regulators of tumor growth, FGF2.

15. SUBJECT TERMS
transgenic mice, growth factors, gene knockout, PyVT mammary cancer

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Table of Contents

Fibroblast Growth Factor-2: an Epithelial Ductal Cell Growth Inhibitor that Drops Out in Breast Cancer

Andrew Baird, PhD,
CDMRP-BCRP IDEA Award

1. Introduction

The original premise for this proposal was that many factors that are found in the normal breast are associated with the progression of breast cancer. In many instances, these factors can have different activities depending on where and when they are being produced, what cell or collection of cells they are acting upon and whether they are present in small or large amounts. We proposed to investigate the growth factor called fibroblast growth factor (FGF). Depending on how it is tested in cell culture, it can stimulate cells, inhibit cells or even change how these cells respond to other factors. So what is it really doing in the breast? And is it involved in the progression of breast cancer? To answer these questions, we proposed to turn to a genetic mouse model of breast cancer where mammary tumors develop with very high predictability, at very predictable times after birth and at predictable frequency. The model, called the "PyVT mouse", was created by introducing a cancer-causing gene from polymer virus (PyV) into the genome of the mouse mammary gland. These mice are otherwise normal except that they all get mammary tumors by 60-85 days of age.

Our idea was to ask two very straightforward questions: (1) what happens to the FGF that is naturally found in the mammary gland when these cancers develop? and (2) what happens to these tumors if there is no FGF in the mammary gland? We reasoned that if FGF is involved in the progression of cancers, then changing the levels of FGF may change how cancer develops. The results, we believe would answer our main objective: is the FGF-FGF receptor system a target for breast cancer therapy.

2. Body

This project began with the goal of obtaining each of the genetic models and then crossing them to develop genetic mouse lines in which we could perform experiments and most importantly ask our central question: Is FGF-FGFR a target for new drug discovery? The results to date are mixed and this progress report aims to capture the progress and frustration in developing these lines. We also describe changes that we have had to make in the original directions we proposed in response to the experimental results. Nevertheless the progress is significant and we have a crossed line to characterize the role of endogenous FGF. In this funding period, we generated the genetically modified line, organized the breeding program to acquire data on tumor progression in these genetically-modified animals and tested the protocols to measure different markers of tumor growth and angiogenesis.

Methods.

Much of this years effort has been directed at methods development, technique validation and the establishment of standard operating procedures for animal breeding, genetic analyses, tumor measurements, in situ hybridization and immunohistochemistry.

Mice: All experiments were conducted under the oversight of the Institutional Animal Care and Use Committee of the University of California, San Diego. This project used three strains of mice that were obtained from Jackson labs. A fourth line FGF-OE were found to be too ill to maintain as a crossed line (see preliminary results below) and we felt proceeding with them would confound the results. We focused on the (1) wild-type, (2) PyVT mice developing spontaneous mammary tumors, (3) FGF2-deficient mice. Our initial intent was to cross hemizygous PyVT (t/+) males and FGF-/- females to generate male offspring that are heterozygous for FGF2 and expresses the transgene. PyVT/FGF2+/- males are crossed with female FGF2+/- mice to yield PyVT/FGF2-/- , PyVT/FGF2+/- , and PyVT/FGF2+/+ mice. We also set up procedures to analyze the different genotypes that we would need to identify from tail DNA by slot blot analysis using probes for PyVT and FGF2.

Tumor measurements: Tumors develop most reproducibly in female mice so we restricted our analyses to follow cohorts of female PyVT/FGF2-/-, PyVT/FGF2+/-, and PyVT/FGF2-/- mice and evaluate mammary tumor onset, incidence, growth and progression. After weaning, body weights of the mice were recorded weekly and the presence of palpable lesions in the mammary glands were determined. Blinded assessments were done with calipers to measure tumor size in two dimensions. Tumor volumes were calculated using a formula of

axb2/2 with a being the length and b the length. Following excessive weight loss or the presence of tumors in excess of 20 mm in length, the mice were killed. Tumor volumes at various time points and tumor weights at necropsy were compared between the three groups using ANOVA followed by a Wilcoxon-Rank test.

Immunohistochemistry: To further characterize mammary tumor development in the absence of FGF2, we performed histological characterization of primary tumors at the early stages of tumor development. Mouse mammary fat pads (MFP) were obtained following euthanasia, perfused with PBS and then fixed with 4% paraformaldehyde (PFA) in PBS, pH 7.4. At the time of immunohistological (IHC) staining, paraffin sections were first deparaffinized in xylene and in progressively more dilute solutions of ethanol. Following this, sections were incubated with Proteinase K (Millipore Cat # 21627 0.2 mg/ml) for 10 minutes. These sections were then blocked in normal goat serum (ABC Rabbit Kit PK-4002) for 1 hour and incubated with either anti-Factor VIII (Biocare) or anti-FGF2 and anti-FGFR1, R2, R3 and R4 (Sigma) at concentrations of 1:100 overnight at 4 C. After washing, sections were then incubated with biotin-conjugated secondary antibody for 30 minutes at room temperature. Between each of the following steps, three separate washes were conducted for 3 minutes each. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in distilled water before the sections were treated with Avidin Biotin Complex (ABC) kit (Vectastatin, Burlingame Ca). For visualization, the sections were incubated with diaminobenzidine substrate for 30 minutes. Following washes, the sections were successively counterstained by incubating in, Hematoxylin, 2% acetic acid, bluing reagent, with separate washes between. Sections were then dehydrated in solutions of progressively more concentrated ethanol and xylene. The cover slips were mounted with Vectamount Mounting Solution. Images were taken with an Olympus FXS100-BSW microscope.

Preliminary results

1. Procurement of mouse lines with altered FGF expression and the PyVT induced tumor to control genetic drift: There is no formal data to show for this aim but it was a major accomplishment. We secured strains of mice and tissues for analyses from Jackson Labs and Douglas Coffin's laboratories respectively to begin the matched breeding and to stabilize genetic drift in what would be a mixed 129-C57/Bl colony that would be selected for breeding. This turned out to be a significant challenge in its own right for the reasons that the PyVT causes cancers in female as they approach breeding age meaning that lines must be maintained through males and het strategies (see breeding below). There were also health issues as illustrated by the deleterious effects of FGF overexpression (see Supporting Data, Figure 1S). In discussions with colleagues, we recognized early in the first year of funding that we had under-appreciated the need for backcrossing and the dramatic misleading effects of outcross studies on tumor onset like when FVB mice are bred into mice with a C57 genetic background. The definitive study on the effects of background on tumor onset in the PyVT model was a study published by Martin et al. which we used as a reference for work going forward although the reviewers of our original proposal were not concerned. We first established that there was a pathologically relevant palpable tumor and onset of the PyVT mammary tumor within 75 days of birth. This is specifically consistent with Martin et al. This paper and the additional references cited below show examples of similar genetic crosses of the PyVT into C57/129-based KO models that have been used to great impact on the field of breast cancer research. Together they have helped define function in genetic models. We recognized that backcrossing 7-9 generations yields the best possible genetic homogeneity when the goal is an ideal genetic inbred model. Not all scientists agree that this is necessary when the wild type controls are performed, as we have done. In the first year of funding we spent considerable effort to observe consistency and appropriate tumor onset after 3 generations of backcrossing in wild type animals. This allowed us to proceed to the 2nd year of proposed studies. Some also believe that crossing knockout mice from a 129 or C57 strain into the FVB MMTV-PyVT should never be done. But such crosses have been done routinely by many groups over the years, and published in rigorous peer-review journals (see attached references). It is also worth noting that our experience with this model for backcrossing is based on discussions with Guy and Muller (Genes and Development 1994), Liao and Johnson (Cancer Research 2007) and Versteeg and Ruf (Cancer Research 2008). Accordingly, three generation backcrossing are sufficient and sibling controls adequate to describe a pathophysiologically relevant onset of mammary cancer in the PyVT model. WE conclude that we have a clear procurement strategy for creating the necessary lines.

2. Breeding: **Establish breeding colony of matched mice for cross over strains and tissue harvesting.** As described above we had significant discussion regarding the breeding strategies when it became apparent that the genetic backgrounds of PyVT and FGF-KO mice were in 129 and C57 backgrounds (see section 1). That backcrossing strategy is described above. We also found in the course of line expansion that the PyVT mice generated tumors at and around their peak of fertility (65 days). This made it very difficult to expand lines through female PyVT mice and a an alternative strategy was developed in consultation with our animal quarters. The approach we used is described in Figure 1 for the FGF-KO and the strategy for the FGF OE mice is presented in Supporting data 2. Briefly, we obtained two founder lines of mice which were (refer to Figure 1 below) the Founder 1 FGF–KO mouse "A1" (FGF-/- and PyVT-) in C57 background and the Founder 2 PyVT mouse "A2" (FGF+ and PyVT+) in the 129 genetic background. These were bread to the 4 expected lines (B1 and B2) of which the heterozygous PyVT+ and FGF-/+ females were identified by gene screen (see below). The M/F ratios were exactly as expected and the distribution of gene knock out was Mendelian. We then proceeded to the C(1) cross of female and male "HETS" (i.e. PyVT+/- and FGF +/-) while discarding the C2 male wild type mice generated (FGF+ and PyVT+). The M/F ratios were again exactly as expected and the distribution of gene knock out was mendelian. The female C1 HETS were selected that were PyVT- so as to permit fertility and respect standards of animal husbandry and they generated the D1 cross that were used for all subsequent backcrossing as illustrated by the D2 path. Male het mice (path D3) were not followed for reasons of cost containment and the variability of tumor progression. Experiments were performed with the E path mice. While we used animals immediately to generate preliminary tumor growth curves we backcrossed 3 generation (path D2) in order to generate genetically clean lines of FGFKO-PyVT mice. WE conclude that we have a comprehensive breeding program.

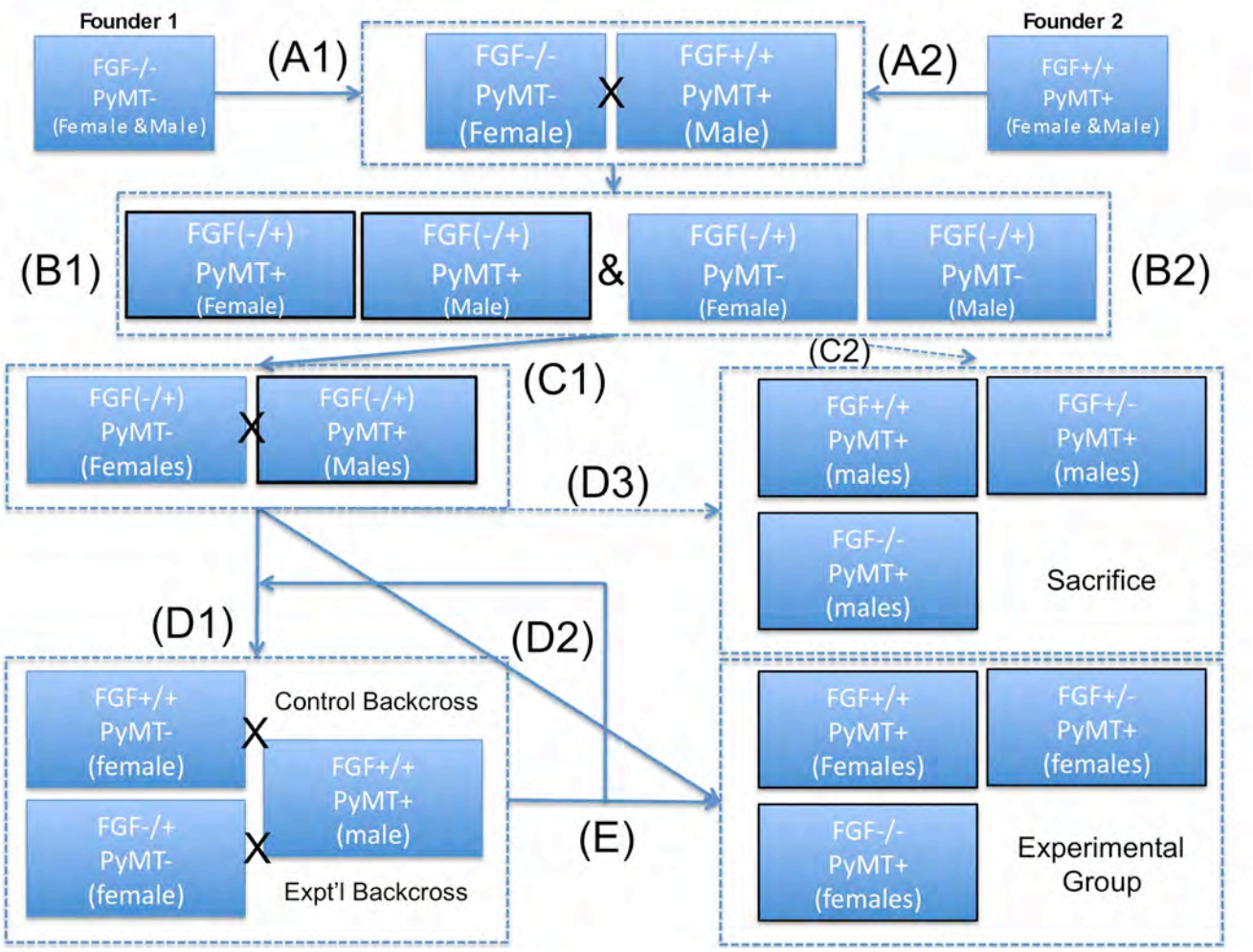

Figure 1: Breeding flow diagram for FGFKO-PyVT mice. As explained in the text founder animals A1 and A2 were bred to the experimental group using the criteria that only PyVT het mice could be used to carry pregnancy. The backcrossing strategy was used for over 3 generations to ensure genetic comparability. Because het siblings were also monitored the effects of gene knock out were followed in siblings as the best controls for genetic drift.

3. Detection of PyVT and FGF transgene expression. We used PCR to validate the detection and screening of the breeding program described above. Two screens were developed. One monitored the presence of the PyVT gene. In the example shown in Figure 2, there are positive bands for PyVT (top panel) but not all are present for FGF (bottom panel). It is possible to use the PCR approach to determine whether both copies of the gene are present or whether there is a single or no copy. These assays were used on all animals generated to classify them as either WT (PyVT+), Het or KO or OE. We did not concern ourselves with the single or two copy of PyVT gene although we kept track of the copy status of FGF. We conclude that detection is possible and can be implemented

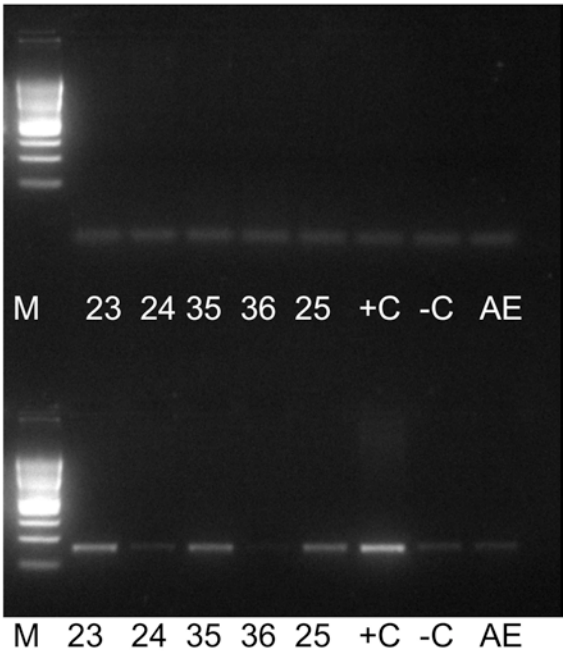

Figure 2: PCR for PyVT and FGF. Tails from 8 samples were analyzed for the presence of either the PyVT or FGF gene as illustrated by the bands observed in the upper panel we would conclude that it is present while in the bottom it is present in 7 of 8 samples like WT, reduced like in a HET mouse in 3 more and presumed absent in the 8th (#36).

4. Immunostaining: In order to be ready for immunostaining & immunoblotting mammary tissues for FGF2 & FGFRs we developed protocols for both. A representative result from an array is shown in Figure 3. We preformed panels of conditions including methods that enhance antigen retrieval like citrate and microwave or using commercial optimization kits. An illustration of these differences are presented in Figure 3 where we show treating sections with Citrate for 10 minutes (Panel A) or proteinase K (Panel B). These could be then processed without and with further amplification. Alternatively we evaluated the use of fluorescently labeled antibodies (e.g. Alexa 488). We also tried multiple antibody concentrations and multiple secondary antibody concentrations. WE conclude that staining needs more optimization and improved technique.

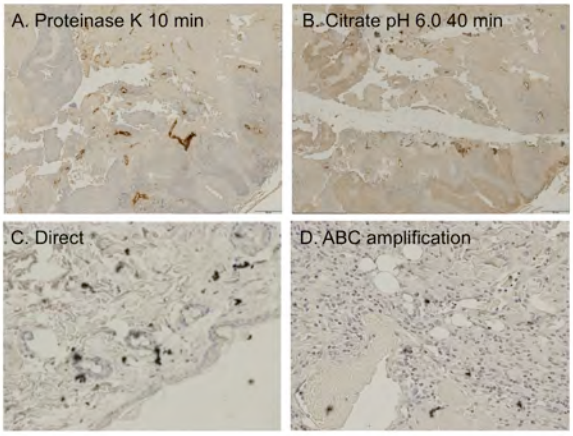

Figure 3: Staining of tumor tissues. Tumors were collected from some of the earliest mice generated in order to get the staining techniques up and going. Shown here are some steps taken for eventual optimization including the citrate pre-wash (Panel A) the proteinase K digestion (Panel B) or alternatively the normal (Panel C) and ABC (Panel D) signal amplification techniques.

5. In situ hybridization probes: Probes for their eventual deployment for in situ hybridization were prepared from plasmids and biotin labeled using the GE kit. We found this method to be reliable, reproducible and applicable to the different expression probes so long as the RNA was expressed with a T4 promoter. The sense was generated to serve as controls

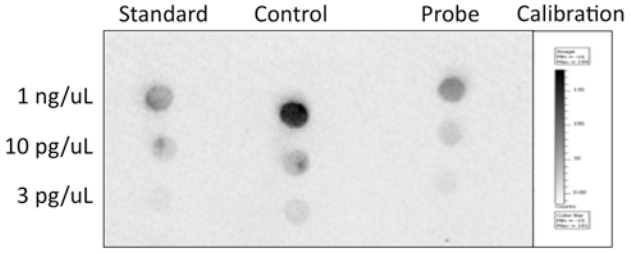

Figure 4: Probe labeling for in situ hybridization cDNA probes were biotinylated and dot blotting used to determine whether it was possible to visualize the 1ng, 10pg or 3 pg of probe. Standard was provided in the kit to calibrate signal, the control to show that the reaction was functional and the probe labeled as well as standard but not as well as the control

TASK	TIME	STATUS	SUMMARY
Task 1	Months 1-12	In progress,	staining and in situ techniques for FGF/FGFR in place.
Task 2	Months 6-12	In progress,	staining and in situ techniques for FGF/FGFR in place.
Task 3	Months 6-12	In progress,	staining and in situ techniques for FGF/FGFR in place.
Task 4	Months 6-12	In progress,	staining and in situ techniques for FGF/FGFR in place.
Task 5	Months 12-24	Not started	animals not mature for experiment.
Task 6	Months 12-24	Not started	animals not mature for experiment.
Task 7	Months 1-36	Initiated,	tumor monitoring/measurements on founder lines.
Task 8	Months 18-36	Initiated,	tumor monitoring/measurements on founder lines.
Task 9	Months 18-36	Not started	animals too sick for basal tumor growth revise breeding.
Task 10	Months 12-24	Initiated,	tumor monitoring/measurements on founder lines.
Task 11	Months 12-36	Initiated,	tumor monitoring/measurements on founder lines.
Task 12	Months 12-36	Not started,	husbandry not possible, revise breeding startegy.

3. Key Research Accomplishments (bulleted list of important research findings resulting from the achievement of project milestones)

- staining methods have been put into place
- Both PyVT and FGF-KO mice have been secured, lines expanded and successfully bred.
- PyVT and FGF-KO mice have been secured and successfully interbred to generate Heterozygous animals.
- Male het and female het mice have been secured and successfully interbred to generate Homozygous line.
- Back-crossing for genetic homogeneity underway to monitor tumor development.

4. Reportable Outcomes (published or in-press manuscripts, abstracts, presentations, products, patents, grant funding awarded or applied for, and career developments that resulted from this award during the reporting year)
None

5. Conclusion
The new mice lines are now in place for validation of the hypothesis in the experimental models of breast cancer. We encountered two unanticipated hurdles. General healthy of FGF++ precludes breeding as cross with tumor generating line and the appropriate controls fro genetic screening require 3-9 back cross generations. Research strategy is being adapted to generate valid results and focus on back crossed breeding program in year 2 (see references below).

6. References
1. Berger T, Cheung CC, Elia AJ, Mak TW. Disruption of the Lcn2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. Proc Natl Acad Sci U S A 2010;107:2995-3000.
In this paper these authors demonstrate that by backcrossing PyMT mice in the FVB into the C57BL/6 background used to generate the lcn-/- mice, they were able to obtain pathophysiologically significant data. Although we did not cross as extensively as this group had done, we were aware of this consideration, and backcrossed to a level sufficient to obtain predictable tumor onsets.
2. Cho SG, Wang Y, Rodriguez M, et al. Haploinsufficiency in the prometastasis kiss1 receptor gpr54 delays breast tumor initiation, progression, and lung metastasis. Cancer Res 2011;71:6535-46.
In this paper PyMT mice in the FVB background are crossed with Kiss1r-/- mice, where the genetic background of the Kiss1r-/- or the backcrossing details are not clearly stated in the methods. The tumor latency was similar to those we are observing in our studies.

3. Denzel MS, Hebbard LW, Shostak G, Shapiro L, Cardiff RD, Ranscht B. Adiponectin deficiency limits tumor vascularization in the MMTV-PyV-mT mouse model of mammary cancer. Clin Cancer Res 2009;15:3256-64.
In this paper adiponectin knockout mice were generated in C57BL/6 background and PyMT mice were from the Muller group, and presumably FVB, but crossing details are not provided. Note the tumor latency is similar to those we are observing in our studies.
4. Fang WB, Jokar I, Chytil A, Moses HL, Abel T, Cheng N. Loss of one Tgfbr2 allele in fibroblasts promotes metastasis in MMTV: polyoma middle T transgenic and transplant mouse models of mammary tumor progression. Clin Exp Metastasis 2011;28:351-66.
In this paper, authors crossed PyMT in the FVB background with Tgfbr2 KO in a C57 BL/6 background with no apparent details on the backcrossing, although we note that tumor onset was consistent with our findings and those of other studies.
5. Guy CT, Muthuswamy SK, Cardiff RD, Soriano P, Muller WJ. Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. Genes Dev 1994;8:23-32.
In this paper, it is not clear what backgrounds the PyMT and the Src models are in, but I have personally communicated with Guy over the years, and based on these discussions, the PyMT is in the FVB background and the Src in Bl6/129 background.
6. Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res 2007;67:563-72.
In this paper, the authors state the HIF1alpha on C57/B6 was crossed with FVB to yield a FVB/C57/B6. In personal communications with Dr. Johnson, we determined that as long as our backcrossing was sufficient to yield consistent tumor onset, then our studies would be on a solid background.
7. Martin MD, Carter KJ, Jean-Philippe SR, et al. Effect of ablation or inhibition of stromal matrix metalloproteinase-9 on lung metastasis in a breast cancer model is dependent on genetic background. Cancer Res 2008;68:6251-9.
In this paper, PyVT mice in FVB were crossed with C57Bl/6 mice bearing mmp ko mutations. This paper details how a role for MMPs was observed in a rag2-/- immunodeficient C57 Bl/6 background had a phenotype whereas FVB backgrounds had no effect.
8. Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG. Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. J Clin Invest 2009;119:252-66.
In this paper, FAK KO mice in a C57/Bl6 background, that we ourselves have been using from the Beggs group at UCSF were crossed with the FVB strain with PyMT with no apparent details on backcrossing.
9. Versteeg HH, Schaffner F, Kerver M, et al. Protease-activated receptor (PAR) 2, but not PAR1, signaling promotes the development of mammary adenocarcinoma in polyoma middle T mice. Cancer Res 2008;68:7219-27.
In this paper, extensive backcrossing of the PyMT and PAR mutants is described. Through personal communication with Drs. Muller and Ruf who are both in San Diego, we validated our more limited degree of backcrossing, where although this study provides the most detailed description of such crosses to date, the results obtained in terms of general tumor onset in control animals was similar to our study

7. Appendices
None

8. Supporting Data

Figure S1: FGF overexpressing mice are sickly, breed with difficulty and not compatible with tumor assays. As show in this figure, mice that overexpress FGF (FGF+/+) are small and sickly and show a gene-dose dependent inhibition in size that is consistent with the hypothesis that FGF2 acts as a multi-action factor that changes activity depending on location. More importantly however, the basal health status of these animals clearly precluded a cross breeding strategy to create a tumor-prone generating line. This is compounded by the response to FGF-KO which is to delay not increase tumorigenicity. Constraints included their general health, the effects of back crossing and most importantly the validity of any tumor studies in an animal line is already sickly and compromised. To this end, we expanded the FGF-KO analyses to include longer back crossing to generate cleaner data and homogeneous genetic lines for the analyses of tumor growth and progression.

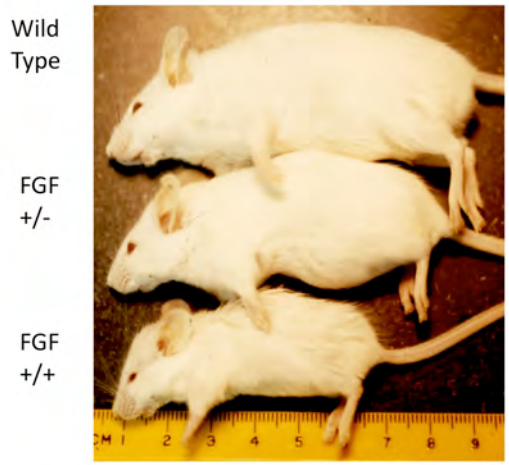

Figure S2: Breeding strategy for FGF OE mice. In the course of the organizational phase of the proposal and in analogy to the breeding program assembled for the FGFKO-PyVT mouse line, we prepared a process for the FGF++ over-expressing mice believing that the females could not be used as carriers but not realizing that their sickly stature and unsuitability for tumor generation made any tumor data generated invalid (see Figure S1). Like with the FGFKO, the founder animals were to be bred to the experimental group using the criteria that only PymT het mice could be used to carry pregnancy. The backcrossing strategy was also to be used for over 3 generations to ensure genetic comparability.